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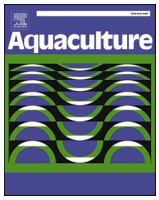

Effect of dietary starch on digestion kinetics and nutrient utilization in yellowtail kingfish (*Seriola lalandi*)

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ABSTRACT

The natural food source of yellowtail kingfish is low in starch, while under commercial farming condition, starch is included at levels >8% to produce extruded diets. In this study, the effect of dietary starch level on digestion kinetics and energy utilization in yellowtail kingfish was investigated. To achieve this, fish were fed equal amounts of crude protein and fat, in combination with either a low amount of starch (*LS* diet; 0% gelatinized wheat flour) or high amount of starch (*HS* diet; 20% gelatinized wheat flour). Each diet was tested in triplicate. Six tanks were stocked with 24 fish (mean initial weight 37 g). Fish performance, digestion kinetics, nutrient digestibility along the gastrointestinal tract and in faeces, and nutrient utilization were evaluated. Kinetics of digestion were altered by starch level, such as dry matter content, osmolality and pH. Viscosity along the gastrointestinal tract was not affected by starch level. High starch level negatively affected the nutrient digestibility in chyme (organic matter, crude protein and starch & sugars) and in faeces (organic matter, crude protein, fat, energy, and phosphorus). In the *LS* diet, the greatest share of organic matter (59.0% absolute digestion) and crude protein (60.8% absolute digestion) was digested already in the proximal intestine, suggesting that the proximal intestine plays the major role in nutrient digestion and absorption yellowtail kingfish are present in the proximal intestine. Despite the fact that fish fed the *HS* diet had a higher carbohydrate and thus energy intake, a tendency for a lower growth was observed. This may be related to the negative effect of high starch inclusion on nutrient digestibility, an increased energy maintenance requirement, the inability of yellowtail kingfish to utilize starch, or a combination.

1. Introduction

Yellowtail kingfish (*Seriola lalandi*) is a carnivorous fish species (Booth et al., 2013). Defining animals as carnivorous is based on the criterion that a minimum of 70% of their natural diet comprises animal tissue (Holliday and Steppan, 2004; Schermerhorn, 2013). In the case of *Seriola* spp., this share of animal tissue surpassed 70%, as fish and cephalopods were identified by stomach content analysis to be their main natural food source (Andaloro and Pipitone, 1997; Pipitone and Andaloro, 1995). Only in a study by Pipitone and Andaloro (1995) on greater amberjack (*Seriola dumerili*), small amounts of non-animal tissue such as pieces of seagrass (*Cymodocea nodose*) and dead leaves were reported in the stomachs. Accordingly, the natural food source of yellowtail kingfish can be considered low in carbohydrate content, although some animal tissues contain carbohydrate fractions such as glycogen or chitin (Abdel-Ghany and Salem, 2020; Ringø et al., 2012; Teng et al., 2001).

In commercial yellowtail kingfish farming, diets contain carbohydrates in concentrations of at least 8% (Horstmann et al., 2023b; Moran et al., 2009). Carbohydrates in the form of starch are added to maintain the structural integrity of (extruded) feed pellets (Romano and Kumar, 2019). Moreover, carbohydrates can be used as an energy source by fish,

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depending on fish species though (Kaushik et al., 2022). Yellowtail kingfish are less efficient in digesting starch (approximately 70%–90%; Horstmann et al., 2023a, 2023b) compared to other finfish species such as rainbow trout (*Oncorhynchus mykiss*) (>98%) or Nile tilapia (*Oreochromis niloticus*) (>99%) (Amirkolaie et al., 2006; Burel et al., 2000; Krogdahl et al., 2004). It has been observed that increasing dietary starch levels negatively impact the digestibility of other nutrients in yellowtail kingfish (Horstmann et al., 2023c). However, the underlying reasons for the negative effects of high starch inclusion on nutrient digestibility were not investigated. Accordingly, it was of interest in the current study to investigate the digestion kinetics in yellowtail kingfish fed a low and high starch diet. Data on the digestion kinetics in fish are rare (Maas et al., 2021), meaning that analysing the digestion along the gastrointestinal tract enhances the understanding of kinetics of digestion in fish.

Kaushik et al. (2022) mentioned that digested carbohydrates do contribute to energy metabolism in fish. Although, the ability to utilize carbohydrates for growth varies largely among fish species (Kaushik et al., 2022; Phan et al., 2022). For instance, the energy utilization efficiency of digestible carbohydrates in carp (*Cyprinus carpio*) (60%), African catfish (*Clarias gariepinus*) (59%), Nile tilapia (66%) and rainbow trout (70%) are higher compared to snakehead (*Channa striata*) (5%) or barramundi (*Lates calcarifer*) (18%) (Phan et al., 2022; Phan et al., 2021; Phan et al., 2019; Schrama et al., 2018). In the current study, we investigated the effect of dietary starch level on digestion kinetics and nutrient utilization in yellowtail kingfish. To achieve this, fish were fed equal amounts of crude protein and fat, in combination with either a low amount of starch (*LS* diet) or high amount of starch (*HS* diet).

2. Materials and methods

The Central Committee on Animal Experiments (CCD) approved this study on the advice of The Netherlands' Animal Experiment Committee (DEC) (permit no. AVD10400202216106). The experiment was as well approved by Wageningen University's Ethical Committee for Animal Experiments and carried out in accordance with the Dutch law (Act on Animal Experiments). The experiment was conducted at the Aquaculture Research Facility (Carus-ARF) of the Wageningen University and Research (Wageningen, The Netherlands). Fish were kept and handled in agreement with EU-legislation.

2.1. Diets

To investigate the effect of dietary starch on digestion kinetics and utilization of yellowtail kingfish, fish were fed equal amounts of crude protein and fat, in combination with either a low amount of starch (*LS* diet) or high amount of starch (*HS* diet). Accordingly, two experimental diets with varying amounts of gelatinized wheat flour were formulated. The high starch (*HS*) diet was made by diluting the low starch (*LS*) diet with 20% gelatinized wheat flour. The used gelatinized wheat flour contained approximately 75% starch. This resulted in an analysed dietary starch content of 4.4% for the *LS* diet and 19.4% for the *HS* diet. Due to the inclusion of 20% wheat flour, the *HS* diet had a lower crude protein and fat content compared to the *LS* diet (Table 1). To feed equal amounts of crude protein and fat, feeding level was adjusted accordingly (described in section 2.3).

Based on past experience, both diets contained >15% fishmeal to ensure a good palatability and thus a good feed intake. Diets were supplemented with taurine to prevent taurine deficiency. DL-methionine and monocalcium phosphate were added to all diets to ensure a balanced amino acid profile and that phosphorus was not a limiting factor for growth. In both diets, a minimum of 10% fish oil was present to avoid deficiencies regarding essential fatty acids. The analysed nutrient composition and physical pellet quality is given in Table 1. Diets were produced by cold pelleting according to Kals et al. (2019) by Research Diet Services (Wijk bij Duurstede, The Netherlands) at the facilities of the Animal Science Group (Wageningen University, The Netherlands), using a co-rotating double screw extruder (M.P.F.50, Baker Perkins, Peterborough, United Kingdom) with a 3 mm die, resulting in 3 mm sinking pellets. After pelleting, diets were first dried for 2 h at 45 °C, followed by drying at 70 °C for 3 h and afterwards cooled to room temperature. All diets were produced approximately one week prior to start of the experiment and stored at 4 °C throughout the experiment.

Table 1
Diet composition, analysed nutrient content and physical pellet characteristics of the experimental diets.

	Low Starch	High Starch
Ingredients (g/kg)		
Gelatinized wheat flour	–	200
Fish meal	197.05	157.64
Wheat gluten	150	120
Pea protein concentrate	150	120
Soy protein concentrate	150	120
Fish oil	130	104
Monocalcium phosphate	10	8
DL-methionine	4	3.2
Taurine	10	8
Casein	130	104
Pellet binders[a]	50	40
Premix[b]	18.75	15
Yttrium oxide	0.2	0.16
Analysed nutrient content (g/kg DM)		
Dry matter (DM, g/kg)	942	926
Crude protein	644	547
Crude fat	175	147
Total carbohydrates[c]	88	230
Starch & sugars	44	194
Non-starch polysaccharides[d]	44	36
Gross energy (kJ/g DM)	23.8	22.8
Crude ash	93	76
Phosphorus	13.5	11.1
Calcium	9.2	7.5
Physical pellet characteristics[e]		
Hardness (N)	107 ± 7	124 ± 12
Durability (%)[f]	99.7 ± 0.0	99.9 ± 0.0
Bulk density (g/L)	575 ± 1	649 ± 2
Gelatinization degree (%)	52	82

[a] Pellet binders – in house composition.
[b] Premix composition. Vitamins (IU or mg/kg complete diet, *HS* diet): Vitamin B1–15 mg; Vitamin B2–15 mg; Vitamin B6–15 mg; Vitamin B5–50 mg; Vitamin B3–150 mg; Biotine – 0.7 mg; B12–0.05 mg; Folic acid – 3 mg; Vitamin C – 500 mg (given as ascorbic acid C, phosphate); Vitamin E – 100 IU; A-vitamin A palmitate – 10,000 IU; D-Rovimix D3–500–2500 IU; K_3 K-menadione sodium bisulphite (51%) – 15 mg; Inositol – 450 mg; Betaine – 500 mg; Choline (given as choline chloride) – 1000 mg; Anti-oxidant BHT (E300–321) – 100 mg; Calcium propionate – 1000 mg. Minerals (mg/kg complete diet; *HS* diet); Iron (as $FeSO_47H_2O$) – 50 mg; Zinc (as $ZnSO_47H_2O$) – 80 mg; Cobalt (as $CoSO_47H_2O$) – 0.2 mg; Copper (as $CuSO_47H_2O$) – 8 mg; Selenium (as Na_2SeO_3) – 0.2 mg; Manganese (as $MnSO_47H_2O$) – 30 mg; Magnesium (as $MgSO_47H_2O$) – 750 mg; Chromium (as $CrCl_36H_2O$) – 1 mg; Iodine (as $CaIO_36H_2O$) – 2 mg.
[c] Total carbohydrates content (on DM basis) was calculated as: 1000 – (crude protein + crude fat + ash).
[d] Non-starch polysaccharides content (on DM basis) was calculated as: total carbohydrates – (starch + sugars).
[e] Presented values are means ± standard deviation.
[f] *Durability expressed as 100% – % feed fines.*

2.2. Fish, rearing conditions and housing facilities

The experiment was conducted over a period of 35 or 36 days. An additional day was needed to accommodate the labour intensive sampling. During each sampling day (day 35 or day 36), six tanks were sampled. Yellowtail kingfish (*Seriola lalandi*) of mixed sex were obtained from a commercial fish farm (Kingfish Zeeland, Kats, The Netherlands).

At the beginning and the end of the experiment, fish were sedated (benzocaine 0.5 mL/L) and batch weighed to determine initial and final weight, and growth. One day prior to stocking and initial weighing, fish were not fed. Per tank, 24 fish with an average initial weight of 37 g were randomly stocked. All fish tanks (370 L water volume, circular) were part of the same RAS, and connected to a sump, settling tank, drum filter (HDF801-1P; Hydrotech, Vellinge, Sweden; mesh size 30 μm), protein skimmer, and trickling filter. Each tank was equipped with an air stone for emergency aeration. The system's refreshment rate was adjusted to keep the NO_3-N concentration below 100 mg/L. The water flow over each tank was controlled (Magnetic-inductive flow sensor, SM 6000; ifm electroic, Essen, Germany) and kept constant at 7.0 ± 0.05 L/min. The outlet of each tank (bottom drain) was directly (~1 m flow distance) connected to an individual swirl separator (column height 44 cm; diameter 24.5 cm; ScaleAQ, Trondheim, Norway) equipped with glass bottles to quantify feed spillage after feeding and to collect faeces.

Pre-set water quality parameters are presented in Supplementary Table S1. Water quality parameters were measured daily from the common outflow. The pH ranged between 7.1 and 7.8, water temperature was 23.8 ± 0.1 °C and salinity 33.5 ± 1.1 ppt during the experimental period. The dissolved oxygen concentration in the tanks' outflow water was always above 5.0 mg/L. Maximum values for TAN (total ammonium nitrogen, NH_4-N + NH_3-N; Merck Aquamerck Colorimetric Ammonium test), NO_2-N (Merck Aquamerck Colorimetric Nitrite test) and NO_3-N concentrations (Merck MQuant Nitrate test strips) were < 0.8 mg/L, <1 mg/L, and < 90 mg/L, respectively. The photoperiod was set at 20 L:4D during the entire duration of the experiment. Light went on at 7:30 am and off at 3:30 am.

2.3. Feeding

During the experimental period, the dietary treatments were tested in triplicate and randomly assigned among 6 tanks. Fish were fed on a pair-feeding scheme as following: The *HS* diet group was aimed to be fed at 23.75 g/$kg^{0.8}$ BW/d (restricted) which is approximately 95% of the predicted satiation level. The *LS* group received approximately 19 g/$kg^{0.8}$ BW/d (approximately 80% of the ration given to the *HS* group), so that every group received the same amount of protein and fat. To maintain an equal amount of feed given per fish per day on crude protein and fat basis for both treatments, the dietary crude protein level was analysed prior to the experiment. Adjusting the feeding level to maintain same crude protein and fat intake caused a lower gross energy intake (approximately 16%) for the *LS* group compared to the *HS* group, being related to the lower carbohydrate content. Throughout the experiment, the daily amount of feed was gradually increased based on the average initial fish weight and the expected daily growth assuming a FCR of 0.9 for the *HS* treatment. The daily amount of feed was divided into two equal portions, which were hand fed at 9:00 h and 15:00 h. During the first four feeding moments of the experiment, the feeding level gradually increased until the intended feeding level was reached. This allowed the fish to adapt to the diet and to prevent feed spills. Fifteen minutes after finishing feeding, the glass bottles attached to the swirl separators were checked for feed pellets to determine and compensate for feed spillage and to avoid feed contamination during faeces collection. Mortality was checked twice a day before feeding.

2.4. Sampling

In this experiment, swirl separators (column height 44 cm, diameter 24.5 cm; hydraulic surface load 213 $m^3/m^2/d$; ScaleAQ, Trondheim, Norway) equipped with glass bottles were used for the collection of faeces. After a four week adaptation period, faeces for digestibility analysis were collected during a 5 day period overnight (15,30 h – 8,30 h) (Amirkolaie et al., 2005). To avoid feed contamination during faeces collection, 15 min after feeding, uneaten feed pellets were quantified and removed from the glass bottles prior to faeces collection. Bottles, which were connected beneath the swirl separators, were submerged into ice water to minimize bacterial degradation of the faecal sample. Faecal samples were pooled per tank and stored at −20 °C until further analysis. During the final sampling days (day 35 and day 36), fish were fed 5 h prior to sampling to be able to collect chyme from all compartments of the gastrointestinal tract. The 5 h before sampling were selected based on a pre-examination investigating the chyme distribution along the gastrointestinal tract, where yellowtail kingfish were fed 3 h, 4 h, 5 h, 6 h, and 7 h prior to sampling. Six tanks were sampled per day. All fish per tank (n = 24) were anesthetized in their tank with benzocaine (0.5 mL/L) and batch weighed afterwards. From those fish, three fish were randomly selected to draw blood from the caudal vein to measure the pH, glucose, osmolality (from serum) and haematocrit (n = 3). Moreover, livers were collected and pooled from these 3 fish to calculate the hepatosomatic index (HSI). Afterwards, all fish were killed with a benzocaine overdose (1.5 mg/L, n = 24). From 21 fish, chyme was collected quantitatively from four segments of the gastrointestinal tract – stomach, proximal, middle and distal intestine. The segmentation of the intestine was based on morphological differences as shown in the Supplementary Image S1. The collected chyme was pooled per tank per segment and stored in plastic containers. Small amounts of chyme were subsampled for osmolality and viscosity measurements. The 21 fish (without chyme, but including viscera) were collected for body composition analysis. Feed samples were taken by pooling 100 g per experimental diet per week and samples were stored at 4 °C.

2.5. Analysis

Faeces collected for digestibility were dried at 70 °C until constant weight. Thereafter, faeces were ground (mixer mill, IKA A11 basic). Frozen fish samples (−20 °C) were ground twice using a meat mincer (TW-R 70; Feuma Gastromschinen GmbH, Gößnitz, Germany) with a 4.5 mm die. Samples for dry matter, ash and crude protein determination were taken from fresh fish samples. Samples for crude fat and energy were freeze dried prior to analysis. Feed, faeces and fish were analysed as described by Staessen et al. (2020). For dry matter determination, feed, faeces and fish were analysed gravimetrically by drying for 4 h at 103 °C until constant weight (ISO 6496, 1999). Ash was determined gravimetrically by combustion for 4 h at 550 °C in a muffle furnace (ISO 5984, 2002) until constant weight. To determine dietary P, Ca, other minerals (feed, faeces and fish) and yttrium (feed and faeces) content using ICP-OES (NEN 6966, 2005 and NPR 6425, 1995), the ash fraction was dissolved in concentrated sulphuric acid by autoclaving (121 °C, 20 min). Total N of the diet, faeces and fish was determined according to Kjeldahl's method (ISO 5983-2, 2009). Crude protein was calculated with a protein conversion factor of 6.25. Crude fat was determined gravimetrically using acid hydrolysis (Hydrotherm®, C. Gerhardt GmbH & Co. KG, Königswinter, Germany) followed by petroleum-ether extraction (Soxhlet method; ISO 6492, 1999). Total starch and gelatinized starch were analysed to determine the gelatinization degree of starch in the experimental diets (Nutrilab, Giessen, The Netherlands). Total starch was analysed enzymatically using amyloglucosidase after washing with 40% ethanol to remove sugars. Gelatinized starch was analysed according to the modified glucoamylase method described by Zhu et al. (2016). For digestibility calculations, starch & sugar content of pelleted diets and faeces was analysed as described above for total starch analysis, leaving out the ethanol washing step. Gross energy of feed, faeces and fish was determined using bomb calorimetry (C7000, IKA werke, IKA analysentechnik, Staufen, Germany).

Collected chyme samples were analysed for dry matter, ash, minerals, yttrium, crude protein, starch & sugars, viscosity and osmolality. Viscosity and osmolality were measured within one hour after sampling on the supernatant (10,000 ×g for 10 min) of fresh samples. Viscosity was measured with Brookfield LVDV ± cone/plate viscometer (Brookfield Engineering laboratories, Middleboro, United States of America). All viscosity measurements were carried out at 24 °C and a shear rate of

50 s^{-1} to 100 s^{-1}. Viscosity was expressed in centipoise (cP) at a shear rate of 100 s^{-1}. Osmolality was measured with Advanced®Model 3320 Micro-Osmometer (Advanced Instruments, Norwood, United States of America). Prior to analysis of minerals, yttrium, crude protein and starch & sugars, chyme samples were freeze dried and ground (mixer mill, IKA A11 basic). Dry matter, minerals and yttrium were analysed as described for feed, faeces and fish samples. Chyme crude protein was analysed according to Dumas method (Ebeling, 1968), using a protein conversion factor of 6.25. Total starch content was analysed according to the total starch assay (AOAC Method 996.11, Amyglucosidase/α-Amylase; Megazyme, Wicklow, Ireland).

Within one hour after sampling, haematocrit was measured from the blood and osmolality and pH was measured from the blood serum. Haematocrit of blood was measured as the percentage of red blood cells compared to total blood volume after 10 min at 10,000 ×g. Serum osmolality was measured on the supernatant with Advanced®Model 3320 Micro-Osmometer (Advanced Instruments, Norwood, United States of America) after centrifugation at 10,000 ×g for 10 min. Serum pH was measured with SenTix SP-DIN (WTW-pH 325). For glucose analysis in blood, serum samples were analysed with the Glucose LiquiColor kit (HUMAN, Wiesbaden, Germany).

Pellet hardness (in N) was tested using a hardness tester (KAHL Pellet Hardness Tester; AMANDUS KAHL GmbH & Co. KG, Hamburg, Germany). Durability (100% - % feed fines) was determined by sieving a 200 g sub-sample through a sieve (1 mm mesh size; 2 min sieving time, interval of 6 s, amplitude of 2 mm/'g'; Retsch, AS 200 control, Haan, Germany). Bulk density was determined with a 1 l cylinder with slide, fall weight and filling cylinder.

2.6. Calculations and data analysis

Growth (g/d) was calculated as $(W_f - W_i)/t$, where W_f is the final body weight, W_i the initial body weight and t is the number of days of the experimental period. Specific growth rate (SGR; %/d) was calculated as $(\ln W_f - \ln W_i) / t \times 100\%$. The absolute feed intake (FI_{abs}; g/d) was calculated as FI_{tot} / t, where FI_{tot} is the total feed intake (g DM). Feed conversion ratio (FCR) was calculated on dry matter basis (g DM/g) as $(FI \times Diet_{DM} / 1000) / (W_f - W_i)$, where $Diet_{DM}$ is the dry matter content of the feed (g/kg). Survival (%) was calculated as $(1 - ((N_i - N_f) / N_i)) \times 100$, where N_i is the number of fish at the beginning and N_f the number of fish at the end of the experiment. Hepatosomatic index (%, HSI) was calculated as $100 \times L_{W3} / W_{F3}$, where L_{W3} is the total liver weight of fish ($n = 3$) and W_{F3} the total final fish weight (n = 3).

Digestibility was calculated for overnight collected faeces but also for chyme per intestine segment. Apparent digestibility coefficient (ADC, %) of organic matter, crude protein, crude fat, carbohydrate, starch, gross energy and minerals were calculated according to Cheng and Hardy (2002) using yttrium as inert marker: $ADC (\%) = 100 \times (1 - ((Y_{diet} / Y_{faeces/chyme}) \times (N_{faeces/chyme} / N_{diet})))$, where Y is the inert marker percentage of the diet, faeces or chyme and N is the nutrient or mineral percentage (or kJ/g gross energy) of the diet, faeces or chyme. Organic matter (g/kg DM) and total carbohydrates in feed, faeces and chyme were calculated as 1000 – ash and as 1000 – (crude protein + crude fat + ash), respectively.

Relative water fluxes (mL/g DM feed intake) per segment were calculated according to (Harter et al., 2013). Nitrogen (N), phosphorus (P) and energy (E) balances are described in the supplementary data. Energy intake, digestible energy intake and daily energy retention were determined in more detail regarding the energy originating from crude protein, fat and carbohydrates. The conversion coefficients were 23.6 kJ/g DM for crude protein, 39.5 kJ/g DM for fat and 17.2 kJ/g DM for carbohydrates (National Research Council (NRC), 2011). Since differences in energy retention could be related to 1) differences in maintenance energy ($E_{Maintenance}$) requirements, 2) differences in the ability to utilize carbohydrates effectively and/or a combination of it, findings on E balances were synthesized in Fig. 5 in the discussion. In situation 1 (Fig. 5a), it is assumed that differences in maintenance energy ($E_{Maintenance}$) resulted in difference in energy retention, while equal energy retention efficiencies were assumed (average of the *LS* & *HS* group). The respective maintenance energy for fish fed the *LS* or *HS* diet were calculated as $E_{Maintenance} = E_{MEI} - ((RE_{CP} / 0.5) + ((RE_{Fat} / 0.9))$, where E_{MEI} is the metabolizable energy intake, RE_{CP} and RE_{Fat} the retained energy as crude protein and fat, respectively. In this calculation, an energetic utilization efficiency of MEI for protein gain of 50% and for fat gain of 90% was assumed (Lupatsch et al., 2003). In Situation 2 (Fig. 5b), differences in retained energy were assumed to be related to differences in energy retention efficiencies, while assuming equal $E_{Maintenance}$ requirements (average of the *LS* & *HS* group). The respective energy retention efficiencies for fish fed the *LS* or *HS* diet were calculated as $RE/DE = RE / (DEI - E_{Maintenance\ LS+HS})$, where RE the retained energy, DEI is the digestible energy intake, and $E_{Maintenance\ LS+HS}$ is the average $E_{Maintenance}$ of both treatments.

2.7. Statistical analysis

Tanks were used as the experimental unit ($n = 6$). A one-way ANOVA was used to investigate the effect of dietary starch level. Statistical significance was tested at a probability level of $p < 0.05$. Values between 0.1 and 0.05 ($0.1 > p > 0.05$) were defined as close to statistical significance and as indicative for tendencies in the data. Statistical analyses were performed by using the statistical program SPSS Statistics 28 (IBM, New York, United States of America).

3. Results

3.1. Fish performance, hepatosomatic index and blood parameters

Fish performance, hepatosomatic index and blood parameters are presented in Table 2. No mortality occurred. Crude protein and fat intake was equal among treatments (as intended), while the *HS* diet resulted in a higher carbohydrate intake by 211% compared to the *LS* diet. The *HS* diet tended to reduce absolute growth (g/d; $p < 0.1$), but no significant effect on SGR was found ($p > 0.1$). A lower FCR was observed for fish fed the *LS* diet (FCR 0.66) compared to the *HS* diet (FCR 0.82) ($p < 0.001$). No effect of starch level was observed on the hepatosomatic index and blood parameters ($p > 0.1$).

Table 2
Fish performance, liver and blood parameters of yellowtail kingfish fed the experimental diets restrictively (3 replicates).

	Low Starch	High Starch	SEM	*p*-value
Fish performance				
Survival (%)	100	100	–	–
Initial body weight (g)	37	37	1.6	ns
Final body weight (g)	180	176	4.2	ns
FI_{abs} (g DM/d)	2.61	3.11	–	–
FI_{CP} (g/d)	1.68	1.70	–	–
FI_{Fat} (g/d)	0.46	0.46	–	–
FI_{CHO} (g/d)	0.23	0.72	–	–
Growth (g/d)	3.93	3.78	0.050	#
SGR (%/d)	4.37	4.24	0.081	ns
FCR	0.66	0.82	0.005	***
Liver and blood parameters				
Hepatosomatic index (%)	1.44	1.35	0.157	ns
Blood haematocrit (%)	43.9	44.2	1.18	ns
Blood pH	7.26	7.25	0.037	ns
Serum osmolality (mOsm/L)	410	414	3.1	ns
Serum glucose (mmol/L)	9.29	9.21	0.728	ns

FI_{abs} – absolute feed intake; FI_{CP} – crude protein intake; FI_{Fat} – crude fat intake; FI_{CHO} – carbohydrate intake; FI_E – energy intake; SGR – specific growth rate; FCR – feed conversion ratio (on DM basis). Values are means and standard error of the means (SEM); ns - not significant $p > 0.1$; # - tendency $p < 0.1$; *** - $p < 0.001$.

3.2. Chyme characteristics and relative water fluxes

Chyme characteristics (dry matter, osmolality, viscosity and pH) and relative water fluxes are visualized in Fig. 1. The data is presented in Supplementary Table S2. Dry matter content of the chyme in the stomach ($p < 0.1$, tendency), proximal ($p < 0.1$, tendency) and middle intestine ($p < 0.05$) was lower for fish fed the *HS* diet compared to fish fed the *LS* diet. The *HS* diet resulted in a higher chyme osmolality in the stomach but reduced the osmolality in the proximal intestine ($p < 0.01$). Chyme viscosity was unaffected by dietary starch level ($p < 0.05$). Fish fed the *HS* diet showed a higher pH in the chyme of the proximal ($p < 0.1$, tendency) and distal intestine ($p < 0.05$) compared to fish fed the *LS* diet. Chyme pH in the stomach and middle intestine were unaffected by dietary starch level ($p > 0.05$). No dietary treatment effect was observed for the relative water influx in the stomach ($p > 0.05$). Fish fed the *LS* diet had a higher relative water reabsorption in the proximal intestine compared to fish fed the *HS* diet ($p < 0.05$). A lower relative reabsorption in the middle and distal gut ($p < 0.05$) was observed for the *LS* diet compared to the *HS* diet.

3.3. Digestibility

In Fig. 2 and Supplementary Table S3, the cumulative progression of digestion (organic matter, crude protein, and starch & sugars) alongside the gastrointestinal tract is shown. The *HS* diet did not affect the organic matter ADC in the stomach ($p > 0.05$), but resulted in a reduced digestibility in the proximal, middle and distal part of the intestine, and faeces ($p < 0.05$) compared to the *LS* diet. Protein digestibility in the stomach tended to be lower for fish fed the *LS* diet compared to fish fed the *HS* diet ($p < 0.1$), while crude protein ADC in the proximal and middle intestine, and in the faeces was higher for fish fed the *LS* diet ($p < 0.01$). In comparison to the *HS* diet, the *LS* diet resulted in a higher digestibility of starch & sugars in the stomach, proximal, middle and distal intestine ($p < 0.05$), while tending to be lower in the faeces ($p < 0.1$). In general, the *LS* diet showed the highest organic matter and crude protein digestibility from the stomach towards the proximal intestine, while the nutrient digestion in the middle was relatively less and even negative in the distal intestine. In comparison, fish fed the *HS* diet had a more gradual digestion from the stomach towards the middle intestine.

Apparent digestibility coefficients (ADC, %) of organic matter, crude protein, crude fat, energy and phosphorus measured in faeces were lower for fish fed the *HS* diet compared to fish fed the *LS* diet (Table 3, $p < 0.05$). Starch & sugar digestibility in the faeces tended to increase for fish fed the *HS* diet ($p < 0.1$). Mineral availability along the intestine is provided in the Supplementary Table S4.

3.4. Yttrium content along the gastrointestinal tract

The collected yttrium (in % of yttrium fed) along the gastrointestinal tract and non-collected yttrium 5 h after feeding is presented in Fig. 3. Equal amounts of yttrium were fed among treatments (*LS* diet 8.16 mg vs. *HS* diet 8.04 mg; $p > 0.1$). Percentage of collected yttrium in the stomach was not affected by dietary starch level ($p > 0.1$). Compared to the *HS* diet, fish fed the *LS* diet tended to have a higher yttrium percentage in the proximal intestine (*LS* diet 8% vs. *HS* diet 3%; $p < 0.1$). Yttrium percentage in the middle (*LS* diet 7% vs. *HS* diet 1%; $p < 0.01$) and distal intestine (*LS* diet 3% vs. *HS* diet 1%; $p < 0.05$) was higher for fish fed the *LS* diet. Fish fed the *LS* diet tended to have a smaller percentage of non-collected yttrium (*LS* diet 62% vs. *HS* diet 81%; $p < 0.1$).

3.5. Energy (E) utilization

E intake, digestible E intake and daily E retention is presented in Fig. 4. E intake from crude protein and fat was equal among diets ($p > 0.05$; Supplementary Table S7). Fish fed the *HS* diet (12.3 kJ/d) had a greater E intake from carbohydrates compared to fish fed the *LS* diet (4.0 kJ/d; ($p < 0.001$). This reflected in a higher total E intake by 14% for fish fed the *HS* diet ($p < 0.001$; Fig. 4a). Digestible E intake from crude protein (tendency, $p < 0.1$) and fat ($p < 0.01$) was higher for fish fed the *LS* diet. Overall, the *HS* diet resulted in a higher total digestible E intake for fish fed the *HS* diet (59.6 kJ/d) compared to fish fed the *LS* diet (56.1 kJ/d; $p < 0.01$; Fig. 4b). Differences in E intake or digestible E intake did not reflect in E growth from crude protein, fat or total E growth ($p > 0.05$; Fig. 4c). Body ash content was higher at fish fed the *HS* diet compared to fish fed the *LS* diet ($p < 0.05$). None of the other body composition parameters were affected by dietary starch level ($p > 0.05$) (Supplementary Table S5). Nitrogen (N), phosphorus (P) and energy (E) balances are expressed on individual fish bases are shown in the supplementary data (Supplementary Table S6). Increasing starch level tended to decrease the digestible N intake ($p < 0.1$), which is a consequence of the lower protein digestibility at fish fed the *HS* diet. Other than the digestible N intake, starch level did not affect parameters of the N balance ($p > 0.1$). Fish fed the *HS* diet had equal protein and fat intake but a higher carbohydrate intake compared to fish fed the *LS* diet. Consequently, gross E intake was higher for fish fed the *HS* diet ($p < 0.001$). Also, digestible E intake and metabolizable E was higher for fish fed the *HS* diet compared to the *LS* diet ($p < 0.01$). In contrast, retained E was numerically lower for fish fed the *HS* diet compared to fish fed the *LS* diet ($p > 0.1$). A higher heat E and E maintenance was observed with higher starch level ($p < 0.01$). Due to the lower digestible E intake, but equal retained E, fish fed the *LS* diet (RE/DE 43.5%) had a 13.9% higher E efficiency compared to fish fed the *HS* diet (38.2%) ($p < 0.05$).

4. Discussion

The aim of the current study was to assess the impact of dietary starch level on growth and nutrient digestion in yellowtail kingfish, in particular how additional starch affects the digestion kinetics. Moreover, this study aimed to explore the capacity of yellowtail kingfish to digest and utilize starch. To achieve this, fish were fed equal amounts of crude protein and fat, in combination with either a low amount of starch (*LS* diet) or high amount of starch (*HS* diet).

4.1. Kinetics of digestion

According to literature, absorption of nutrients is not anticipated to occur in the stomach (Uys and Hecht, 1987; Xing et al., 2023). However, in the current study, the organic matter, crude protein and starch & sugar ADC in the stomach was on average 8.9%, 17.0% and 47.0%, respectively (Fig. 2). Similar crude protein digestibilities were observed for Nile tilapia and rainbow trout (Ciavoni et al., 2023b; Ciavoni et al., 2023a; Maas et al., 2021). An observed nutrient digestion in the stomach may be related to the solubility of proteins and starch & sugars, which could have resulted in a quicker transition of these nutrients to the proximal intestine compared to yttrium as suggested by literature (Allen et al., 2007; Ciavoni et al., 2023b; Harter et al., 2015; Xing et al., 2023). The higher observed digestion of starch & sugars (47.0%) from the stomach compared to proteins (17.0%) supports this statement, as starch & sugars are more water soluble compared to proteins (Allen et al., 2007). Moreover, the decrease in starch & sugar ADC from the stomach towards the proximal intestine by 18.7% (absolute) at the *HS* diet can as well be seen as an indicator for an unequal progression of nutrients and yttrium (Fig. 2). An unequal progression of nutrients compared to yttrium may as well be promoted by nutrient sensing driven peristalsis, as hypothesized by Ciavoni et al. (2023b). Overall, this raises the question if yttrium is a good marker to investigate the kinetics of digestion in yellowtail kingfish.

Research in rainbow trout showed a gradual increase in crude protein digestion from the stomach towards the middle intestine (Ciavoni et al., 2023b; Ciavoni et al., 2023a). In contrast, research by Verdile et al. (2020) suggested that the digestive and absorptive functions are not equally distributed along the intestinal length in rainbow trout. In

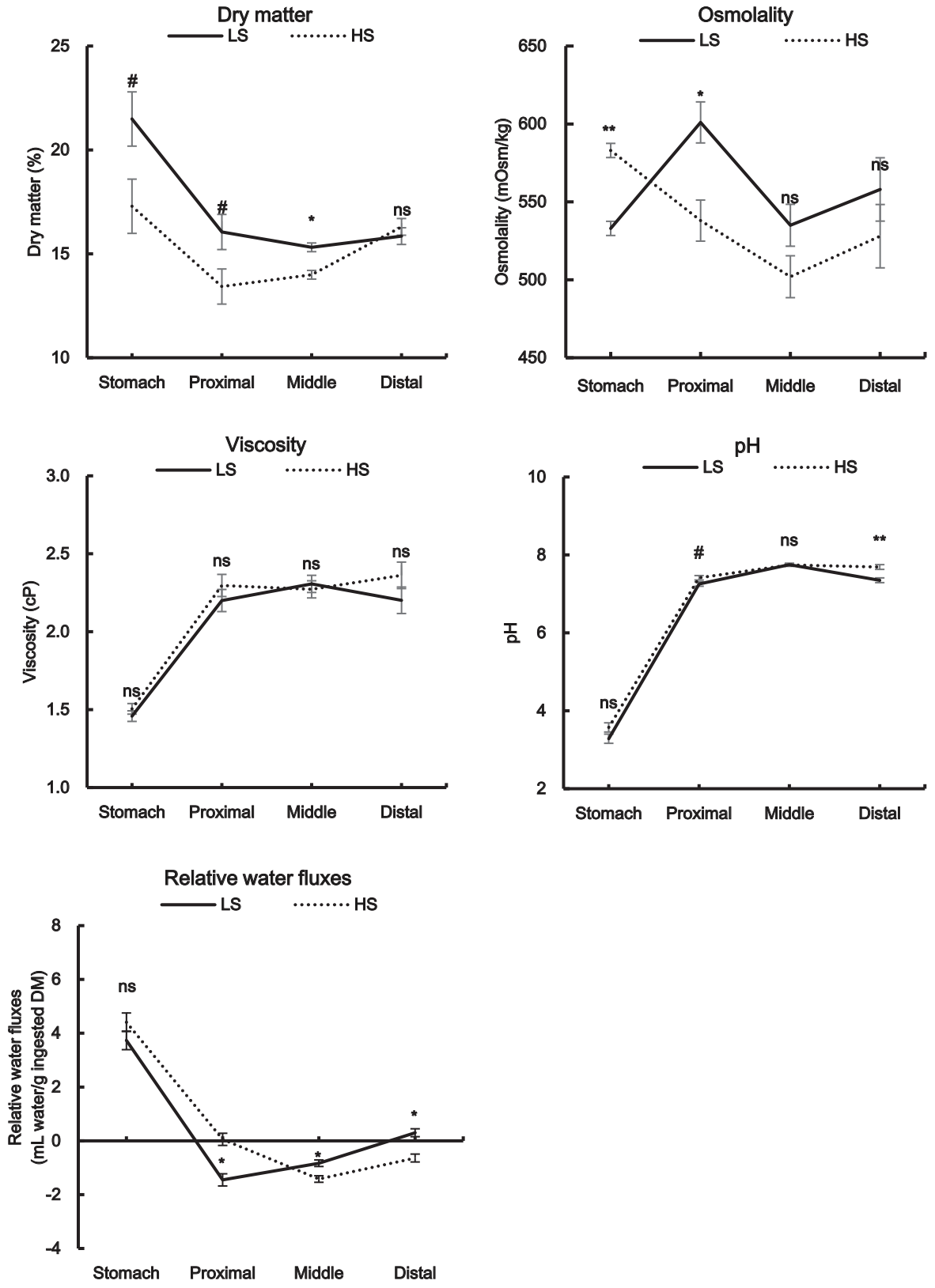

Fig. 1. Chyme characteristics (dry matter, osmolality, viscosity and pH) and relative water fluxes measured in the stomach, proximal, middle and distal intestine in yellowtail kingfish fed the experimental diets restrictively for 35–36 days; LS – Low starch; HS – High starch. Values are means and standard error of the means (SEM); ns - not significant $p > 0.1$; # - tendency $p < 0.1$; * - $p < 0.05$; ** - $p < 0.01$.

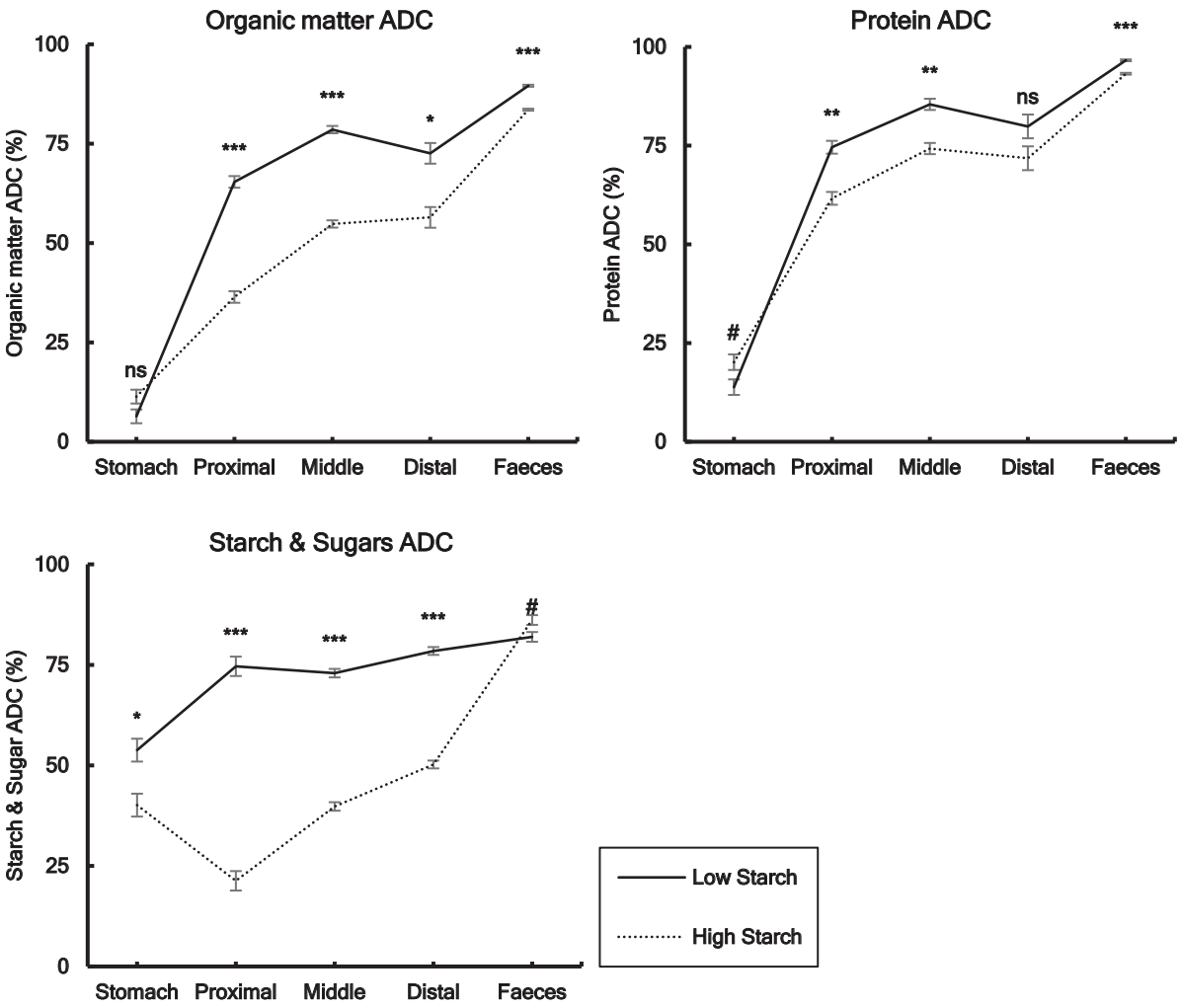

Fig. 2. Progression of digestion (ADC) of organic matter, protein and starch & sugars measured in the stomach, proximal, middle and distal intestine, and in the faeces from yellowtail kingfish fed the experimental diets restrictively; Values are means and standard error of the means (SEM); ns - not significant $p > 0.05$; * - $p < 0.05$; ** - $p < 0.01$; *** - $p < 0.001$.

Table 3
Apparent digestibility coefficient (ADC, %) measured in faeces of yellowtail kingfish fed the experimental diets restrictively (3 replicates).

	Low Starch	High Starch	SEM	p-value
Organic matter	89.6	83.5	0.25	***
Crude protein	96.6	93.2	0.09	***
Crude fat	91.0	80.5	0.53	***
Starch & sugars	82.0	86.2	1.21	#
Energy	91.8	86.1	0.22	***
Phosphorus	65.6	62.2	0.57	*

Values are means and standard error of the means (SEM); # - tendency $p < 0.1$; * - $p < 0.05$; *** - $p < 0.001$.

the current study, fish fed the *HS* diet had a gradual progression of digestion for organic matter and crude protein from the stomach towards the middle intestine and for starch & sugars from the proximal towards the distal intestine (Fig. 2). In contradictory, for fish fed the *LS* diet, the greatest share of organic matter (59.0% absolute digestion) and crude protein (60.8% absolute digestion) was digested in the proximal intestine, while digestion in the middle intestine (organic matter 13.2% and crude protein 10.9% absolute digestion) was relatively lower. Accordingly, it can be concluded that dietary starch inclusion alters the digestion rate in the proximal intestine in yellowtail kingfish, while such effects were absent in studies with rainbow trout fed diets with

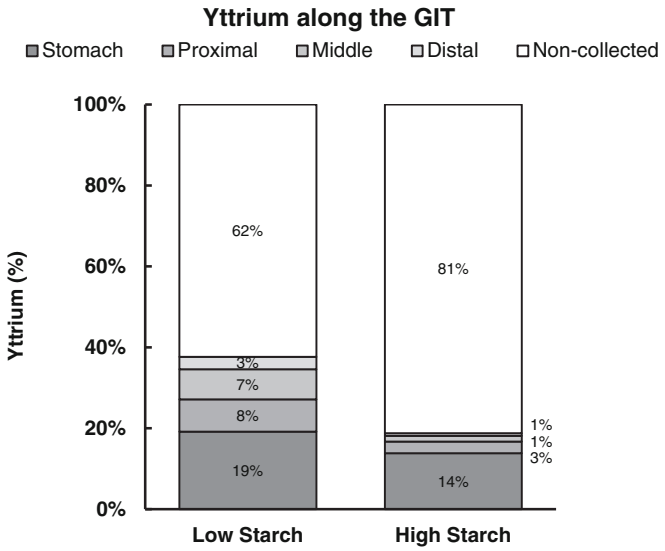

Fig. 3. Yttrium content (in %) along the gastrointestinal tract (GIT) and non-collected yttrium of yellowtail kingfish fed the experimental diets restrictively for 35–36 days. Values are means.

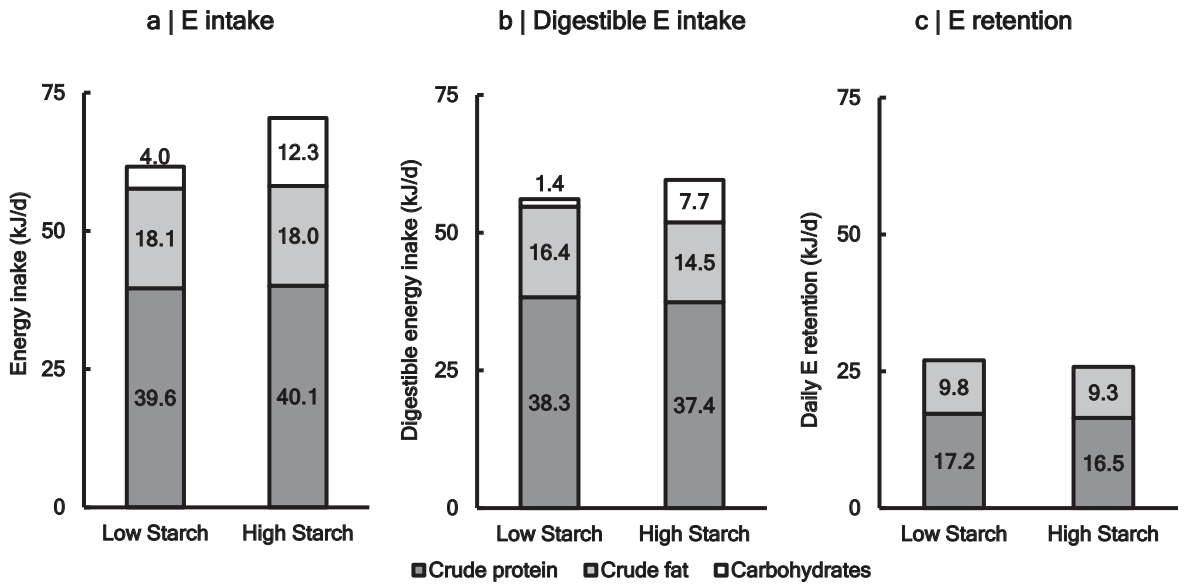

Fig. 4. Energy intake (in kJ/d; $p < 0.001$), digestible energy intake (in kJ/d; $p < 0.001$) and daily energy retention (in kJ/d; $p > 0.05$) of yellowtail kingfish fed the experimental diets restrictively (3 replicates).

contrasting starch content (Ciavoni et al., 2023a); Ciavoni et al., 2023b). Moreover, our findings raise the question whether a gradual digestion from the stomach towards the middle intestine is natural in yellowtail kingfish as the natural food source of yellowtail kingfish does not contain large amounts of carbohydrates (Andaloro and Pipitone, 1997; Pipitone and Andaloro, 1995). Since the *LS* diet is in regard to its nutrients closer to a natural diet of yellowtail kingfish compared to the *HS* diet, it can be hypothesized that the digestion pattern of the *LS* diet reflects the natural digestion kinetics in yellowtail kingfish better. Based on this, it could be hypothesized that the majority of the digestive and absorptive functions in yellowtail kingfish are present in the proximal intestine.

Previous research has shown that high dietary starch inclusion negatively affects the nutrient digestibility in yellowtail kingfish (Horstmann et al., 2023c) which is largely in line with the current study. However, in regard to the crude fat digestibility, a greater negative effect of high dietary starch inclusion was observed in the current study (absolute −10.5%) compared to the previous study (absolute −4.0%). In the current study, it was in particular of interest to investigate the effects of high starch inclusion on digestion kinetics. As previously mentioned, it was clearly shown that the largest differences in nutrient digestibility among treatments occurred in the proximal intestine (Fig. 2), while in the middle and distal intestine absolute differences in nutrient digestibility remained or became slightly smaller. Based on literature, an increased inclusion of dietary starch can lead to increased moisture content in the stomach (Amirkolaie et al., 2006; Elesho et al., 2022; Harter et al., 2015; Harter et al., 2013) and an increased viscosity of the chyme (Amirkolaie et al., 2006; Harter et al., 2015). Harter et al. (2015) observed in African catfish negative effects of high dietary starch level on crude protein digestibility and suggested that this was related to an increased viscosity, while stomach dry matter content was excluded as a cause. In general, elevated viscosity can result in a reduced mixing of the chyme and creation of a layer (barrier) between absorptive cells and chyme in the lumen, thereby reducing the digestibility of crude protein (Harter et al., 2015; Tran-Tu et al., 2019, 431). However, in the current study, the *HS* diet did not alter the viscosity (Fig. 1), which contradicts with the results observed by Amirkolaie et al. (2006) in Nile tilapia and the results and conclusion observed by Harter et al. (2015) in African catfish. In the current study, it is hypothesized that the higher moisture content in the stomach, proximal and middle intestine lead to the observed lower nutrient digestibility in fish fed the *HS* diet (Fig. 1). According to literature, moisture content can affect the gut transit time, whereby a higher stomach moisture content shortens the gut transit time (Harter et al., 2015; Ruohonen et al., 1997). To further illustrate, research by Ruohonen et al. (1997) has shown that a lower feed moisture content appears to extend stomach emptying and increase gastric emptying time in rainbow trout. This is in line with the current study, as the *HS* diet tended to increase the moisture content in the stomach, proximal and middle intestine, resulting in less yttrium in the gastrointestinal tract (Fig. 3). A lower percentage of yttrium and thus chyme in the gastrointestinal tract of fish fed the *HS* diet could indicate a faster gut transit time for fish fed the *HS* diet. However, our results on yttrium content along the gastrointestinal tract have to be treated with care, as the non-collected yttrium does not necessary equals the excreted yttrium. Quantitative chyme collection is difficult and thus may result in an under estimation due to incomplete stripping. Furthermore, in the current study the pyloric caeca were not stripped. So part of the non-recovered yttrium might have been remained in the pyloric caeca. Moreover, yttrium might have a different progression along the gastrointestinal tract as nutrients (e.g. protein or carbohydrates) as indicated beforehand. This could as well have resulted in observed differences among diets. But most likely the large difference in non-collected yttrium is due to a shortened transit time induced by starch supplementation. Besides that, differences in gut transit time could also be partly related to the higher absolute feed intake in fish fed the *HS* diet (3.11 g DM/d) compared to fish fed the *LS* diet (2.61 g DM/d) (Table 2). However, similar negative effects of the *HS* diet on faecal nutrient digestibility were observed during a previous experiment with yellowtail kingfish when equal amounts of dry matter were fed (Horstmann et al., 2023c).

4.2. Nutrient utilization

Fish fed the *HS* diet (70.4 kJ/d) had a higher energy intake compared to fish fed the *LS* diet (61.7 kJ/d) (Fig. 4), whereas a tendency for a lower growth was observed for fish fed the *HS* diet (Table 2). This lower growth rate, despite equal protein and fat intake, can be partly attributed to the observed lower crude protein and fat digestibility in fish fed the *HS* diet (Table 3) which is discussed in detail above. Besides that, fish fed the *HS* diet had a lower energy retention and a significantly lower energy efficiency compared to fish fed the *LS* diet (Supplementary Table S6). The lower energy retention in fish fed the *HS* diet could be

related to: 1) an increased maintenance energy ($E_{Maintenance}$) requirement (Fig. 5a), 2) the inability of yellowtail kingfish to utilize carbohydrates effectively (Fig. 5b), and/or a combination. In the first case (*Situation 1*), it would be hypothesized that the *HS* diet resulted in an increased $E_{Maintenance}$ requirement, while equal energy retention (RE) efficiencies (Eq. 1). In the second case (*Situation 2*), it would be hypothesized that the *HS* diet resulted in a reduced energy efficiency, while equal $E_{Maintenance}$ requirements (Eq. 2).

$$\text{Equation 1}: \quad E_{Maintenance} = E_{MEI} - ((RE_{CP}/0.5) + ((RE_{Fat}/0.9)) \tag{1}$$

where E_{MEI} is the metabolizable energy intake, RE_{CP} and RE_{Fat} the retained energy as crude protein and fat, respectively. In this calculation, an energetic utilization efficiency of MEI for protein gain of 50% and for fat gain of 90% was assumed (Lupatsch et al., 2003).

$$\text{Equation 2}: \quad RE/DE = RE/(DEI - E_{Maintenance\ LS+HS}) \tag{2}$$

where RE the retained energy, DEI is the digestible energy intake, and $E_{Maintenance\ LS+HS}$ is the average $E_{Maintenance}$ of both treatments.

Situation 1: Hypothesizing that the *HS* diet resulted in an increased maintenance energy requirement, while expecting equal energy efficiencies, a significantly higher $E_{Maintenance}$ requirement was observed for fish fed the *HS* diet (16.3 kJ/d) compared to fish fed the *LS* diet (10.0 kJ/d; $p < 0.001$; Fig. 5a and Supplementary Table S6). A higher observed maintenance requirement could be related to the higher feed intake at fish fed the *HS* diet, thus, resulting in a higher energy expenditure for feeding, digestion and absorption of nutrients. Moreover, a higher maintenance requirement could be based on increased vital life processes, such as swimming behaviour, protein turnover rate, tissue repair or oxygen uptake rate.

Situation 2: In *Situation 1*, the $E_{Maintenance}$ was based on energetic utilization efficiency coefficients for protein gain (50%) and fat gain (90%) according to Lupatsch et al. (2003). This dependency on specific coefficients introduces a potential source of error, which could lead to errors in the observed findings. It could be as well hypothesized that the maintenance energy requirement was not different among treatments, while expecting different energy efficiencies among diets (Fig. 5b). In this case, a significantly higher retained energy per digestible energy was observed for fish fed the *LS* diet (0.57 kJ RE/DE) compared to fish fed the *HS* diet (0.49 kJ RE/DE; $p < 0.01$; Fig. 5b). According to research by Phan et al. (2021) with snakehead, an inability to utilize carbohydrates can be related to a limited capacity to regulate plasma glucose levels and limited storing capacity (e.g., in the form of triglycerides). However, both plasma glucose levels (Table 2) and body fat content (Supplementary Table S5) were not affected by dietary starch level in the current study. This means that energy was lost elsewhere e.g., in the form of glucose via the branchial and urinary pathway. However, glucose losses via the branchial and urinary pathway are not reported in fish. In summary, this study showed that the addition of starch in form of 20% gelatinized wheat flour did not improve fish growth; even a tendency for a lower growth was observed in fish fed the *HS* diet (Table 2). Whether this negative effect of the *HS* diet can be sorely assigned to negative effects on nutrient digestibility, an increasing energy maintenance requirement, the inability of yellowtail kingfish to utilize starch, and/or a combination, could not be answered. To answer this question, further research is required on carbohydrate utilization, in particular starch & sugar utilization, in yellowtail kingfish.

5. Conclusion

High dietary starch level altered kinetics of digestion and negatively affected the nutrient digestion along the gastrointestinal tract in yellowtail kingfish. The largest differences in nutrient digestion occurred already in the proximal intestine, suggesting that the proximal intestine plays the major role in nutrient digestion and absorption in yellowtail kingfish. The addition of 20% gelatinized wheat flour did not improve fish growth despite a higher energy intake. Even a tendency for a lower growth was observed in fish fed the high starch diet. This negative effect of high starch inclusion on fish growth and energy retention may be related to its negative effect on nutrient digestibility, increasing energy maintenance requirements, the inability of yellowtail kingfish to utilized starch, or a combination.

CRediT authorship contribution statement

P. Horstmann Zuther: Writing – review & editing, Methodology, Investigation, Formal analysis, Data curation, Conceptualization. **Roel M. Maas:** Writing – review & editing, Supervision, Methodology, Data curation, Conceptualization. **Tijmen Blok:** Writing – review & editing, Investigation, Data curation. **Jeroen Kals:** Writing – review & editing, Methodology, Conceptualization. **Marit A.J. Nederlof:** Writing – review & editing, Methodology, Investigation, Conceptualization. **Satya Prakash:** Writing – review & editing, Investigation, Formal analysis. **Henk A. Schols:** Writing – review & editing, Methodology. **Thomas W.O. Staessen:** Writing – review & editing, Project administration, Funding acquisition, Conceptualization. **Yaqing Zhang:** Writing – review &

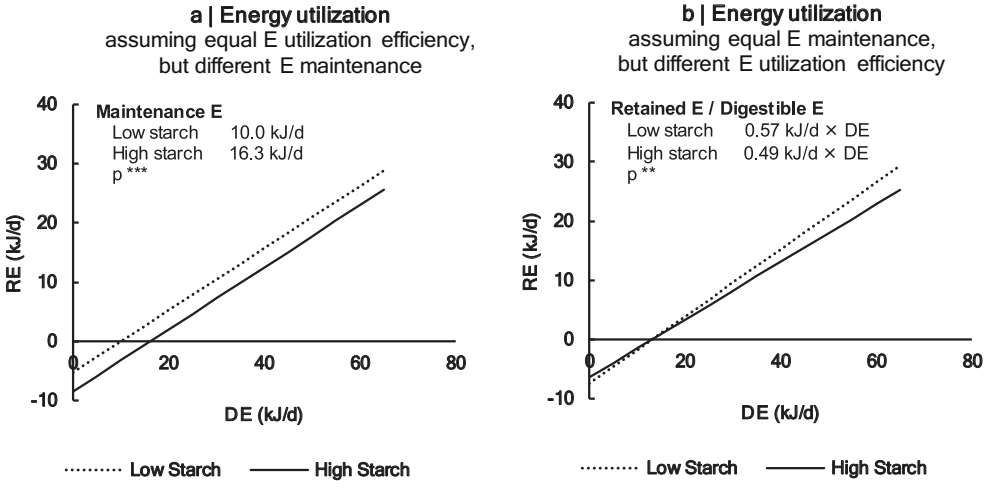

Fig. 5. Energy (E) utilization (in kJ/d) of yellowtail kingfish fed the low starch and high starch diet when either a) assuming an equal energy efficiency (0.52 kJ RE/kJ DE), but different maintenance (graph a; Low starch, RE = 0.52 × DE – 5.2; High starch, RE = 0.52 × DE – 8.5) or b) assuming an equal maintenance (13.1 kJ/d), but different energy efficiency (graph b; Low starch, RE = 0.57 × DE – 7.4; High starch, RE = 0.49 × DE – 6.4). The equal values for the energy efficiency or energy maintenance were the average of the treatments; RE – retained energy; DE – digestible energy; ** - $p < 0.01$; *** - $p < 0.001$.

editing, Investigation. **Fotini Kokou:** Writing – review & editing, Supervision, Conceptualization. **Johan W. Schrama:** Writing – review & editing, Supervision, Project administration, Methodology, Conceptualization.

Declaration of competing interest

This work is part of the Healthy Happy Kingfish project applied for by Kingfish Zeeland B.V. under the subsidy scheme Innovation Projects Aquaculture 2019 and, granted by the RVO (Netherlands Enterprise Agency) under the application number 19111000012. This project is partly funded by The European Union with support of the European Maritime and Fisheries Fund (EMFF).

Data availability

Data will be made available on request.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.aquaculture.2024.741149.

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